Remarks

Claims 1-114 are pending in the application. By this amendment, claims 1-15, 21-55, and 57-114 are canceled, without prejudice, as drawn to a non-elected invention. Claims 17 and 20 amended above in response to a §112, second paragraph indefiniteness rejection set forth at page 8 of the latest Office Action. Upon entry of this amendment, claims 16, 17-20, and 56 will be pending before the Examiner.

The specification has been amended above to obviate the objections set forth in section 6, at page 3 of the Office Action. Applicants are attempting to locate the same information in a printed source at which point and time they reserve the right to amend the specification to explicitly set forth that information.

Next, claims 16-20 and 55 stand rejected under §112, first paragraph, as not enabled. Applicants respectfully traverse. As an initial point in support of the enablement rejection, the Examiner argues at page 5 that, based on Herbers *et al.* (1996), that metabolic control is a function of many enzymes in a pathway and not due to single rate-limiting steps; and further that a metabolically altered transgenic plant will be only beneficial if plant growth and survival are not seriously affected. In response to this point, Applicants acknowledge that biosynthethetic pathways are certainly comprised of many functioning enzymes and thus the CONTROL per se of a specific biosynthesis is generally not viewed in terms of single rate-limiting steps. However, there are numerous examples of up-regulation and down-regulation of a specifically targeted single enzymatic step of a biosynthetic pathway, that is a first committed step of the pathway at the start or of a downstream branching point, that has led to alteration of an accumulated product. One well-known example is for anthocyanin pigmentation in flowers, part of the phenylpropanoid for flavonoid biosynthesis pathway. As reviewed in K. Davies (2000), Metabolic engineering of plant secondary metabolism, Kluwer Academic Publishers, eds R. Verpoorte and A.W. Alfermann, for example pages 133-134 and references therein, enzyme CHS is recognized as the first committed step in flavonoid biosynthesis and DFR is recognized as the first specific step to anthocyanin biosynthesis furthering the pathway such that down regulation of these single enzymes led to an expected reduction in pigmentation.

Regardless, Applicants have chosen to manipulate the PRECURSOR POOL for a biosynthetic pathway rather than enzymatic steps within a biosynthetic pathway itself, even if steps in the metabolic pathway are known to be committed steps and have a track record of successful manipulation. The subject invention provides an enhanced pool of universal precursors for isoprenoid products-this is reflected in the fact that the pending claims concern isoprenoid products for the very reason that these precursors can affect several derivative classes of products depending on the host cell genetics. It is well-known in the art, such as taught in Sandmann *et al.*(1999) and Sandmann (2001), that enhanced supply of the essential 5-carbon precursors IPP and DMAPP will affect positively downstream isoprenoid metabolism in cells with a functional biosynthetic pathway, especially in plants, and that IPP and DMAPP are absolutely required for advances in bioengineering of increased isoprenoids such as carotenoids and other isoprenoid production.

Further, it is known to those skilled in the art that such manipulation commonly utilizes cells with a way for the precursors to be used such that an abundance may not seriously affect cell growth; the logical goal of directed manipulations is to influence specific isoprenoid product accumulation. This is taught in M. Harker and P.M. Bramley (1999) FEBS Letters, Vol. 448, pages 115-119 and in P.D. Matthews and E.T. Wurtzel (2000) Appl. Microbiol. Biotechnol., Vol. 53, pages 396-400 that overexpression of DXP synthase alone and with IPP isomerase in a carotenogenic *E. coli* strain substantially increased lycopene levels without toxicity to the organism. Such an elevation of carotenoids without associated negative effects would not be expected in a non-accumulating strain. This is further taught in plants by S.S. Mahmoud and R.B. Croteau (2001) Proc. Natl. Acad. Sci. USA, Vol. 98, pages 8915-8920, who overexpressed DXP reductoisomerase in peppermint to enhance the yield of essential oil by increasing the plastidial supply of IPP and DMAPP without detrimental effects on the plant. Thus even though the claims do <u>not</u> require an absence of effects on plant growth and survival, ample evidence that increased isoprenoid production can be effected in both microbes and plants without seriously affecting growth and survival.

The second point in support of this rejection, the Office Action asserts at pages 5-6 that, specific to the mevalonate pathway, the art teaches that overexpression of the single enzyme HMG-CoA reductase had some effects on sterol derivatives from the isoprenoid pathway but other isoprenoid products were unchanged in levels, suggesting that regulation of biosynthesis is occurring

in plants (and animals); and further (page 6), the MVA kinase from eukaryotes, such as higher plants and mammals, is regulated by feedback inhibition. In response to this point, Applicants note that the subject invention can affect many different classes of isoprenoids because we influence the pool of universal precursors. Indeed, the particular isoprenoid that is elevated or accumulated can be predicted as it is in peppermint and carotenogenic *E. coli* (see above references). Our invention allows more carbon to enter the isoprenoid pathway and it is known in the art that this will raise the level of some isoprenoid depending on the host cell (again as described above). The Chappell 1995 paper cited in the Office Action showed that increasing merely one element of the mevalonate pathway was indeed capable of directing more carbon into the isoprenoid pathway, underscoring our invention that elevating the IPP supply (with concomitant increase in DMAPP due to presence of either native IPP isomerase, or IPP isomerase resulting from a heterologous IPP isomerase-encoding polynucleotide as described in Examples 10-14 and 16-20 of the specification) can elevate downstream isoprenoids. Indeed it is now generally established in the art that any means to increase supply of the 5-carbon precursor(s) will result in increased flux of carbon in the isoprenoid pathway. This is further proven for higher plants in S.S. Mahmoud and R.B. Croteau (2001), Proc. Natl. Acad. Sci. USA, Vol. 98, pages 8915-8920, in which peppermint was directed to increase yield of essential oil by simple manipulation of DXP reductoisomerase to increase the plastidial supply of IPP and DMAPP without detrimental affects on the plant. It was predicted that in the mint system elevation of essential oil would occur since the cell is predisposed to accumulate that product; control mechanisms in the native cell were thus negligible.

In *E. coli* similar results were seen as to those described and taught in the subject application. Applicants do not claim infinitely elevated levels of isoprenoid production, just increased levels as compared to a non-transformed cell, and this is what occurs in the bacterial cell. Those skilled in the art understand that feedback regulation has a higher chance of setting in once a critical upper limit is reached, even if such physiological controls are unknown at the time. The vast majority of reports to increase IPP/DMAPP precursor supply in cells and higher plants have had the expected outcome of elevated isoprenoid levels, with the specific isoprenoid level depending on the host cell.

It is important to note that the subject invention is fundamentally distinct from prior art that concerns isolated enzymatic elements of the mevalonate pathway (such as HMG-CoA reductase of

MVA kinase). Applicants' invention involves introducing into target cells a polynucleotide for the mevalonate pathway in toto, not just isolated elements. Such cited studies show that the target enzyme increases in total enzyme activity but do not show that increasing TOTAL precursor pathway activity would be subject to the same control mechanisms or feedback inhibition, or would be able to affect accumulation of other pathway classes of products, especially if the pathway is active in a cell or subcellular compartment such as the plastid, that lacks an active mevalonate pathway in its native state as taught by Applicants herein regarding the subject invention.

The truth of Applicants' teachings is confirmed by Martin *et al.* (2003) Nature Biotech, Vol. 21, pages 796-802, in which 1) use of the entire mevalonate pathway was superior to isolated elements in terms of delivering high-level precursors and to cause an increase in flux of the isoprenoid pathway; 2) funneling of the precursor supply into production of a class of terpenoid products consumes the excess of precursor, thereby eliminating any growth inhibition; and 3) engineering of the entire pathway into a cell naturally lacking the pathway circumvents any control mechanisms of the pathway expression since no native regulatory elements are present. Compartmentalization of the enzymes for the entire mevalonate pathway into the microalgae or higher plant plastid mimics the prokaryotic system, and thus no control or feedback mechanisms are present.

As a third attempt to support the enablement rejection, the Office Action at page 6 cites to Cordier *et al.* (1999) as teaching that use of a higher plant cDNA for DPMVA decarboxylase in yeast had unexpected results presumably because the plant enzyme had lower specific activity (lower functioning), in yeast compared to in the plant. In response, Applicants note that it is well known in the art that use of an isolated heterologous enzymatic component may have lower functioning in a particular host, but nevertheless it may demonstrate some activity. It is obvious to anyone skilled in the art that one can substitute one cDNA for another from a different source to affect altered activity in a particular host. However, and as taught by Applicants, insertion of the complete mevalonate pathway has been shown to be fully functional and to affect increased flux in the isoprenoid pathway. See Martin *et al.*, (2003) Nature Biotech, Vol. 21, pages 796-802, with use of the heterologous *S. cerevisiae* mevalonate pathway in an *E. coli* host.

A fourth point mentioned in the Office Action in support of this rejection concerns the use of polycistronic polynucleotides and their asserted unpredictability in plants, particularly as applied to claims 18-20 (see page 6 of the Office Action). The Office Action asserts that the art teaches the expression of such polynucleotides is not predictable because monocistronic operons are the norm in plants, except for in chloroplasts. In response to the assertions regarding polycistronic polynucleotides, Applicants note that their specification teaches insertion of a polycistronic polynucleotide into 1) a prokaryotic host in which polycistronic operons are the norm; and into 2) the plastids (for example, chloroplast) of microalgae and higher plant cells in which polycistronic operons are also the norm based on their prokaryotic origin. Expression of such polycistronic polynucleotides is entirely predictable because this is natural for bacteria and plastids of plant cells as is well known in the art. It is taught in R. Bock (2001) J. Mol. Biol., Vol. 312, pages 425-438, specifically page 430 and references therein, that transforming DNA will integrate into the plastid genome in entirely predictable fashion due to the mechanism of homologous recombination using flanking sequences surround the polynucleotide to recombine with the host plastid genome, and it was taught by Staub and Maliga (1995) Plant J., Vol. 7, pages 845-848 that polycistronic operons will be transcribed untruncated due to the principally prokaryotic mechanisms of gene expression; and it is taught in the review by R. Bock and R. Hagemann (2000) Progress in Botany, Vol. 61, pages 76-90, specifically page 84 (cited in the specification), that the exact tuning of the expression level of transgenes is possible in such vectors in transgenic plastids. Thus our invention is enabled as claimed for cells, and especially for plant cells as in the claims 18-20.

Moreover, premature translation termination, questioned in the Office Action, is <u>not</u> predictably an issue in plants transformed with a polycistronic polynucleotide in the nucleus as opposed to the plastid. This is addressed in a review on strategies for polycistronic or polygenic transgene expression in plants by Hunt and Maiti, 2001 (In Vitro Cell. Dev. Biol. -Plant, vol.37, pages 313-320, specifically pp. 315-317). Use of the viral gene VI mediated the translation of polycistronic messages in nuclear-transgenic plants and provided for coordinated and stable expression levels of different cistrons. This review proves that as of the priority date of this application the state of the art was such that those of ordinary skill in the art expected that a large number of cistrons could be translated in plants using this strategy, with at least six cistrons being

translated in the viral genome. As an alternative, a polyprotein encoded by a fusion polynucleotide can be processed into multiple functional mature polypeptides by including a type of viral protease gene in transgenic plants, with at least eight proteins resulting from this autolysis mechanism in viruses.

Further, Applicants do not teach only polycistronic polynucleotides can be used. Rather, as would be known to anyone skilled in the art in view of Applicants' teachings, a polynucleotide can comprise a series of open reading frames with their own regulatory elements that enable transcription and subsequent protein targeting to the organelle in which it is functional (such as the plastid). Such a polynucleotide can be inserted into the nucleus of a higher plant for transcription. It is taught in M.J. Leech *et al.* (2000) Metabolic engineering of plant secondary metabolism, Kluwer Academic Publishers, eds R. Verpoorte and A.W. Alfermann, pages 69-86, for example, page 78 and references therein that multigene metabolic engineering is enabled via particle bombardment. Nuclear multigene engineering specific to isoprenoid production in plants is further taught in X.D. Ye *et al.* (2000) Science, Vol. 287, pages 303-305, as cited in the specification.

It is known in the art that a combination of polynucleotides, at least up to 11, can be integrated at one genetic locus into one effective polynucleotide through introduction of multiple genes on separate plasmids via particle bombardment and without the need for multiple physically linked selectable markers as described by Hunt and Maiti, 2001 (In Vitro Cell. Dev. Biol. -Plant, vol.37, pages 313-320) and references therein. This is enabled by the subject application using an effective selection agent such as fosmidomycin to greatly simplify the process of identifying among primary transformants those, and only those, that contain the complete functional mevalonate pathway, as described by Applicants in Examples 15, 28, 29 and 20. All other transformants will not survive. This selection process is thus superior to that of the state of the art, as described on page 7 of Applicants' disclosure. This holds true as well as for individuals who possess a complete functional mevalonate pathway as a result of by genetic complementation via sexual hybridization or fusion of cells, as is known in the art. Other examples of expression of several transgenes in plants using a combination of polynucleotides that are monocistronic and linked are given in the review by Hunt and Maiti, 2001 (In Vitro Cell. Dev. Biol. -Plant, vol.37, pages 313-320) and are

considered to be routine in the art since selectable marker or reporter genes are commonly assembled with genes of interest on transformation vectors.

In addition, recent publications by others have confirmed the truth and broad applicability of Applicants' teachings. See, for example, Martin *et al.* (2003) referred to above; WO 02/099095, Lopez-Ulibarri *et al.*, published December 12, 2002; and U.S. 2003/148479, Kim Seon-Won *et al.*, published August 7, 2003. Each of these publications discusses construction of operons containing genes which encode enzymes of the mevalonate pathway, which genes are heterologous to the intended target cells, and which show successful expression in the target organisms of the heterologous polynucleotides. These successes confirm the truth of Applicants' teachings regarding the ability of the skilled artisan to obtain heterologous polynucleotide sequences encoding enzymes of the mevalonate pathway, combine these polynucleotides into a functional operon, and successfully introduce the operon into a target cell, wherein it is expressed.

Finally, the Office Action asserts that Applicants' purported lack of evidence of reduction to practice somehow results in a requirement of undue trial and error (Office Action at pages 6-7). Response, Applicants refer to the publications cited previously which confirmed the truth of Applicants' teachings, and further point to Exhibit A hereto which is proof of an actual reduction to practice of the teachings of Examples 1 through 7 of the subject application. Exhibit A shows map and restriction analysis of Applicants' vector pHKO2, confirming that it contains orfs encoding the complete mevalonate pathway, and confirming that introduction of this vector into target cells resulted in expression of the enzymes of the complete mevalonate pathway, proving that pHKO2 was effective in providing a mevalonate pathway to organisms previously lacking such, resulting in synthesis of IPP from acetyl-CoA, further confirming the truth of Applicants' teachings.

The law requires objective enablement, which may be accomplished by use of illustrative examples, broad terminology, or a combination of both as in the subject application. See *In re Marzocchi*, 169 USPQ 367 (CCPA 1971). Applicants have taught that those skilled in the art should obtain polynucleotides encoding enzymes of the complete mevalonate pathway, construct plasmids containing functional operons thereof, and introduce these plasmids into target cells wherein they will function to produce increased isoprenoid production as compared to non-transformed cells. None of these steps requires undue experimentation. Since the teachings are present, the only

relevant concern of the Patent Office is whether they are true. *In re Marzocchi, supra*, at 369. Applicants' teachings have been proven to be true. The law requires nothing more. Reconsideration and withdrawal of this rejection is respectfully requested.

Finally, claims 17 and 20 stand rejected at page 8 of the Office Action under §112, second paragraph, as indefinite. Applicants respectfully assert that the above amendments to the claims have obviated this rejection, and reconsideration is requested.

Applicants gratefully acknowledge the Examiner's indication that the pending claims are free of the prior art. Applicants believe, that in view of the foregoing, all claims as currently pending are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge to Deposit Account 19-0065 any fees under 37 CFR 1.16 or 1.17 as required by this paper.

Applicants invite the Examiner to call the undersigned if clarification is needed on any of this amendment, or if the Examiner believes that a telephone interview would expedite prosecution of the subject application to completion.

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JL/srp
Attachments: Petition and Fee for Extension of Time; Exhibit A; Copies of the following cited references:

Bock, R. "Transgenic Plastids in Basic Research and Plant Biotechnology" J. Mol. Biol. (2001) 312:425-438.

Mahmoud, S.S. *et al.* "Metabolic engineering of essential oil yield and composition in mint by altering expression of deoyxylulose phosphate reductoisomerase and menthofuran synthase" PNAS (2001) 98(15):8915-8920.

Matthews, P.D. *et al.* "Metabolic engineering of carotenoid accumulation in *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of deosysylulose phosphate synthase" Appl Microbial Biotechnol (2000) 53:396-400.

Sandmann, G. *et al.* "The biotechnological potential and design of novel carotenoids by gene combination in *Escherichia coli*" TibTech (1999) 17:233-237.

Sandmann, G. "Genetic manupulation of carotenoid biosynthesis: strategies, problems and achievements" Trends in Plant Science (2001) 5(7):14-17.